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CULTIVATION OF TUBERCLE BACILLI

E. Louis Goodman and Mary Moore

Research Department, National Jewish Hospital for Consumptives, Denver

The factors, respiratory and nutritional, which influence the growth of the tubercle bacillus in the test tube are numerous and complicated. The literature is burdened with reports on methods to hasten the development of this bacillus both in the culture tube and in the animal body. The true tubercle bacillus, however, persists in growing slowly and a careful analysis of the available studies reveals that there are many sources of misinterpretation, especially when it is reported that it can be grown luxuriantly within a few days. In such cases the organisms are usually not pathogenic and frequently prove to be some other acid-fast organism, the mere property of acid-fastness being used to classify them erroneously as tubercle bacilli. Thus, we have heard that certain animal tissues and other substances incorporated into mediums have an especially hastening influence on the growth of these organisms. In spite of these reports, reliable laboratories continue to grow tubercle bacilli on certain simple standard mediums, i.e., glycerol agar, Dorset's egg medium or modifications thereof, inspissated serums and glycerol broth.[1] When Nocard and Roux [2] found that the addition of glycerol to the medium markedly enhanced the growth of human tubercle bacilli, a decided progress was made, while Dorset's [3] introduction of the egg medium gave the laboratory a universally obtainable and easily preserved material for suitable medium for this bacillus.

In 1915 Petroff [4] described his method for the primary cultivation of human tubercle bacilli from contaminated material, such as sputum and feces, in which the material is given a preliminary treatment with an equal volume of 3% sodium hydroxide solution for 20 to 30 minutes at incubator temperature and, after removal of the alkali the residue seeded on Dorset's egg medium containing a 1: 10,000 dilution of gentian violet to inhibit the development of organisms not killed by the sodium hydroxide. He isolated the tubercle bacillus from 129 of

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[1] Corper, H. J.: Amer. Rev. Tuberc., 1919, 3, p. 461.

[2] Ann. de l'inst. Pasteur, 1887, 1, p. 19.

[3] Amer. Med., 1902, 3, p. 555.

[4] Jour. Exper. Med., 1915, 21, p. 38.

135 sputum specimens.[5] Stewart[6] obtained cultures by Petroff's method from 24 of 37 sputums positive microscopically. Corper, Fiala and Kallen[7] in cultivating 526 microscopically positive sputums obtained macroscopic cultures in only 27.3% (144). At that time no discrepancies could be found in the procedure used by them, and Petroff's high percentage could not be accounted for since the mediums and details of technic used were well controlled.[8] In recent observations by Corper, Gauss and Rensch[9] it was found that carbon dioxide in concentrations as low as 3% can inhibit the growth of tubercle bacilli and that bacilli seeded on glycerol agar contained in fairly large culture tubes (6x1 inches) and subsequently glass sealed and incubated would not grow sufficiently to be visible macroscopically, while the contained gases would reach about 5% carbon dioxide concentration. These observations made it seem highly desirable to study the effect of tin-foil capping on the growth of tubercle bacilli in tubes, it being thought that the tin-foil capping may possibly prevent ready access of air to the cultures and thus the amount of carbon dioxide developed by the bacilli and retained in the tube rise sufficiently to account for some of the negative cultures in the observations cited. In order to study the effect of this part of the manipulation 100 culture tubes (5/8 by 6 inches) similar to those used in the routine cultivation of tubercle bacilli from the sputum, were prepared with a sterile 5% glycerol agar slant, neutral to litmus, sterilized by autoclaving, and 100 tubes of the same size containing a sterile slant of egg medium with 1:10,000 dilution of gentian violet (Petroff), sterilized by inspissation. After inoculation these tubes were divided into groups of 20 of each medium, half of which were stoppered with paraffined cotton and then covered with paraffined cloth to prevent the medium from drying out and to allow easy access of atmospheric air, and the other half stoppered with paraffin cotton stoppers and then carefully covered with tin-foil using every precaution to avoid tears or breaks in the tin-foil and to seal as tightly as possible by using 3 small rubber bands tightly drawn around the top of the culture tube and tin-foil cover. Each set of 20 of one kind of medium was inoculated with a separate culture of tubercle bacilli. The cultures used were 3 human cultures, one old laboratory avirulent strain "human," and two recently isolated virulent human

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[5] Bull. Johns Hopkins Hosp., 1915, 26, p. 276.

[6] Jour. Exper. Med., 1917, 26, p. 755.

[7] J. Infect. Dis., 1918, 23, p. 267.

[8] Personal communication.

[9] J. Amer. Med. Assn., 1921, 76, p. 1216, and Amer. Rev. Tuberc., 1921, 5, p. 562.

strains "Gluckson" and "Weisbrod," and 2 bovine strains, an old laboratory culture "Bov. D" and a recently isolated culture "Bov. V." The tubes were read after one and two months' incubation at 37.5 C. (table 1).

TABLE 1

THE GROWTH OF TUBERCLE BACILLI ON GLYCEROL AGAR AND PETROFF'S MEDIUM IN CULTURE TUBES WITH PARAFFINED AND TIN-FOIL CAPS

Culture	Medium	Tube Sealed with	Number of Culture Tube																			
			Growth One Month After Inoculation										Growth Two Months After Inoculation									
			1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
"Human"	Glycerol agar	P	3	3	3	2	2	2	1	1	0	C	4	4	4	4	4	4	4	4	4	C
		T	3	2	2	2	2	2	1	1	1	0	4	4	4	4	4	4	4	4	4	3
	Pet-roff's	P	3	3	2	2	2	2	2	1	1	1	4	4	4	4	4	4	4	3d	3d	2d
		T	3	2	2	2	1	1	1	1	0	C	4	4	4	4	4	4	4	4	3	C
"Gluckson"	Glycerol agar	P	3	3	3	3	3	3	3	3	3	2	4	4	4	4	4	4	4	4	4	4
		T	3	3	3	3	3	3	2	2	2	2	4	4	4	4	4	4	4	4	4	4
	Pet-roff's	P	3	3	3	3	2	2	2	1	0	0	4	4	4	4	4	4	2	2	1d	0d
		T	3	3	3	3	3	2	2	2	2	0	4	4	4	4	4	3	3	3	2	2
"Weisbrod"	Glycerol agar	P	3	2	2	2	1	1	1	1	0	0	4	4	4	4	4	4	4	3d	3	3
		T	3	2	2	2	2	1	1	1	1	1	4	4	4	4	4	4	4	3	3	2d
	Pet-roff's	P	0	0	0	0	0	0	0	0	0	0	4	4	4	3	2	1	1	0	0	0
		T	1	0	0	0	0	0	0	0	0	0	4	4	4	4	4	3	1	1C	C	0
"Bov. D."	Glycerol agar	P	2	2	2	1	1	1	1	1	1	1	4	4	4	4	4	4	4	4	4	4
		T	2	2	1	1	1	1	1	1	1	C	4	4	4	4	4	4	4	4	4	C
	Pet-roff's	P	3	3	2	2	2	2	2	2	2	1	4	4	4	4	4	4	4	4	2d	2C
		T	3	3	3	3	2	2	2	2	2	2	4	4	4	4	4	4	4	4	4	4
"Bov. V."	Glycerol agar	P	2	2	2	2	2	1	1	1	1	0	4	4	4	4	4	4	4	4	3	3
		T	2	2	2	2	1	1	1	1	1	0	4	4	4	4	4	4	4	4	4	3
	Pet-roff's	P	3	3	3	3	3	3	3	2	2	1	4	4	4	4	4	4	4	4	4	3d
		T	3	3	3	3	3	3	3	3	2	2	4	4	4	4	4	4	4	4	4	4

The tubes in line P were stoppered with sterile cotton plugs dipped in sterile melted paraffine and covered with paraffine cloth held in place by a rubber band.

The tubes in line T were stoppered with sterile cotton plugs dipped in sterile melted paraffin and covered carefully with tin-foil snugly bound with three small rubber bands.

The figures indicate the amount of growth in the culture tube and are graded from "0" indicating no growth to "4" indicating a luxuriant and maximum growth. "C" indicates the culture was contaminated and "d" after a numeral indicates the medium dried up and growth indicated by the numeral is still visible.

Table 1 reveals that tin-foil capping held in place by rubber bands drawn as tightly as possible around the neck of the culture tube planted with tubercle bacilli does not seal these tubes well enough to permit an accumulation of sufficient of the respiratory gases (carbon dioxide) to in any way inhibit or prevent the growth of these bacilli. The tin-foil capping can, therefore, not account for the negative cultures (*72.7%* of positive microscopic sputums) obtained by Corper, Fiala and Kallen.

The tubercle bacillus was originally grown by Koch on inspissated blood serum mediums. Being a surface grower, an aerobe, it does not do so well in liquid mediums, so that unless special purposes are in view, such as the preparation of "old tuberculin," it is usual to grow the bacillus on either an inspissated serum or egg medium or glycerol agar. On glycerol broth prepared in the same way as glycerol agar with the exception that the agar is omitted, the cultures usually require a longer time to become luxuriant and frequent cultural failures are obtained. The factors involved in these differences in the results obtained on the two mediums may be due to the difficulty in obtaining satisfactory surface seeding in the glycerol broth, the tendency of the glycerol broth to form a liquid film about the bacilli preventing satisfactory respiratory exchange, the differences in physical composition of the two mediums and probably other equally important factors. In order to obviate the difficulty of surface seeding in broth cultures a number of suggestions have been made, i. e., to rub the culture on the glass at the liquid level, to use a small amount of serum which has been coagulated at the liquid level on the glass, to put cotton into the medium, etc. Comparative studies on these points are, however, lacking. It is also to be noted that small lumps of tubercle bacilli are more apt to give cultures than fine emulsions. Recently Masucci[10] found that the addition of amino-acids in the form of "aminoids" (Arlington Chemical Co.) to the ordinary glycerol broth markedly increased and hastened the growth of tubercle bacilli on this medium. The glycerol broth he used was prepared from fresh meat juice to which were added 1.7% "Difco" peptone and 0.3% "aminoids." From the standpoint of technic a test of the value of the addition of "aminoids" to broth for growing tubercle bacilli seemed worth while. Two types of "aminoids," beef aminoids with 75% amino-nitrogen, biuret free, and milk (casein) aminoids with 65% amino-nitrogen were used in these experiments. In the first experiment ordinary glycerol broth, prepared from meat juice, peptone and glycerol, neutral to litmus was made and put into 250 c c sterile cotton stoppered nursing bottles in amounts of about 90 c c. To test the value of the beef blood "aminoids" this was added to the broth in 0.3% amounts, after which the reaction was again adjusted to neutrality to litmus. The broth was then put into bottles in 90 c c amounts, the mediums were sterilized by autoclaving and were then planted with an avirulent laboratory "human" strain, and "Gluckson," a virulent recently isolated culture. In the same way 0.3% milk "aminoids" were added to the broth, and the bottles were planted with the tubercle bacilli. The bottles were kept at 37.5 C. for two months with the results recorded in table 2.

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[10] Jour. Lab. & Clin. Med., 1920, 6, p. 96.

TABLE 2

THE EFFECT OF THE ADDITION OF "AMINOIDS" TO GLYCEROL BOUILLON ON THE GROWTH OF HUMAN TUBERCLE BACILLI

Tubercle Bacilli	"Aminoids"	Series	Glycerol Bouillon Culture Flasks 1	2	3
"Human"	Beef	Controls	+++*	+++	+
		Test	—	—	—
	Casein	Controls	++	++	+
		Test	+++	++	—
"Gluckson"	Beef	Controls	+++	+++	++
		Test	—	—	—
	Casein	Controls	+++	++	++
		Test	++	+	

* The culture flasks after inoculation were incubated at 37.5 C. for two months after which the readings were made and recorded. — Indicates no growth and +++ a luxuriant maximum growth covering the entire surface of the broth. The flasks were observed at weekly intervals throughout the period of incubation.

These results do not indicate that the addition of "aminoids" in 0.3% amount to the ordinary glycerol broth in any way hastened the growth of human tubercle bacilli. There would seem to be rather a retarding (as noted with the beef "aminoids") effect on the growth, indicating that there are probably other factors instrumental in determining the rate or luxuriance of growth rather than the mere presence or absence of amino acids in the form of "aminoids." Complete studies of the metabolism and chemistry of the tubercle bacillus, such as are being carried on by Long,[11] and comparative culture rate experiments carefully controlled as suggested by our study will throw more light on the exact conditions necessary for the maximum growth of the tubercle bacillus in different mediums.

SUMMARY

The human tubercle bacillus grows equally well on culture tubes containing slants of glycerol agar or egg medium (Petroff's) regardless of whether the tubes have been capped with waxed cloth or with tin-foil, securely held in place with rubber bands, thus indicating a ready access of atmospheric air in the folds of the tin-foil in spite of precautions to prevent this means of the escape of the carbon dioxide produced by the respiration of the bacilli. Tin-foil capping does not account for the low percentage (27.3%) positive cultures obtained by Corper, Fiala and Kallen in cultivating microscopically positive sputums. The addition of "aminoids" beef or casein to the ordinary laboratory glycerol broth has no appreciable effect on the growth of human tubercle bacilli on this medium; such an addition may even markedly retard the growth of the bacilli as was found to be the case with beef "aminoids."

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[11] Am. Rev. Tuberc., 1920, 4, p. 842.